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Soluble insulin receptor ectodomain is elevated in the plasma of patients with diabetes mellitus.

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Soluble Insulin Receptor Study Group*
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Running title: A new biological marker for monitoring the hyperglycemic state.

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Abstract

"Objectives" Insulin binds to the α-subunit of the insulin receptor (IRα), and subsequently exerts its effects in the cells. The soluble ectodomains of several receptors were found to circulate in the plasma. Therefore, we hypothesized that soluble human IR ectodomain (α-subunit and a part of β-subunit) may exist in the plasma of diabetes patients.

"Research Design and Methods" We identified soluble hIR ectodomain in human plasma by a two-step purification followed by immunoblotting and by a gel-filtration chromatography. Furthermore, we established a hIRα-specific enzyme-linked immunosorbent assay (ELISA) and measured the plasma IRα levels in patients with diabetes mellitus (DM). We also investigated this phenomenon in streptozotocin-induced diabetic hIR transgenic mice.

"Results" The soluble hIRα, but not intact hIRβ or whole hIR, exists in human plasma. The plasma IRα levels were significantly higher in patients with type 2 and type 1 DM than in control subjects (2.26 ± 0.80 (type 2, n=473) and 2.00 ± 0.60 ng/ml (type 1, n=53) vs. 1.59 ± 0.40 ng/ml (control, n=123), $p<0.001$ vs. the control). The plasma IRα level was positively correlated with the blood glucose level and 10-20% of the insulin in patient plasma bound to hIRα. In the *in vivo* experiments using diabetic hIR transgenic mice, hyperglycemia was confirmed to increase the plasma hIRα level and the half-life was estimated to be ~6 h.

"Conclusion" We propose that the increased soluble IR ectodomain level appears to be a more rapid glycemic marker compared to HbA1c or glycoalbumin.

The ectodomains of receptors for several cytokines and growth factors have been found to circulate in the plasma (1-3). In 1972, Gavin et al. demonstrated that an insulin binding protein was shed from the surface of cultured cells (4). Subsequently, Pezzino *et al.* observed a circulating protein that corresponded to the insulin receptor (IR) in healthy human plasma (5). Furthermore, the IR α-subunit (IRα) and IR β-subunit (IRβ) were found to be secreted into the incubation medium by various cultured cell lines (6), and IR shedding from cultured human lymphocytes has been reported (7). Thus, the existence of soluble IR in human serum has been suspected. However, no detailed clinical investigation has yet been carried out. We previously reported that an injection of purified human insulin receptor α-subunit (hIRα) increased the blood glucose level in mice (8). Furthermore, transgenic mice secreting soluble IRα into the plasma showed chronic hyperglycemia (9). Here, we established novel ELISA systems to measure both the ectodomain (α-subunit and a part of β-subunit) of IR and full-length of IR. With these ELISA systems, we report that soluble hIRα with parts of extracellular region of hIRβ, but not as a whole IR or with intact hIRβ, is present in human plasma and that its plasma level is elevated in patients with elevated blood glucose. The ectodomain of IR may be cleaved, at least in part, by hyperglycemic state-associated mechanisms.

Research Design and Methods

Insulin receptor sandwich ELISA systems. We developed two kinds of ELISA systems to specifically measure hIRα and full-length IR, respectively. (See details in Online Appendix)

Study subjects. Control-1; Healthy Japanese volunteers with no diabetic history or familial diabetic history in relatives within the third degree in Tokushima district (N=123) were enrolled as Control-1 and we confirmed their normal glucose tolerance by 75 g oral glucose tolerance according to the World Health Organization (WHO) guidelines for DM (10). Control-2; Healthy Japanese volunteers with no diabetic history or familial diabetic history in Nagano district (N=120) confirmed to have normal fasting plasma glucose (FPG) and HbA1c levels were enrolled as Control-2. Outpatients seen at the Shiga University of Medical Science in Shiga district (T2DM, N=474), the University of Tokushima Affiliated Hospital in Tokushima district (T2DM, N=162) and the Tokushima Univ. Hospital in Tokushima district (T1DM, N=53) were enrolled as T2DM-1, T2DM-2 and T1DM, respectively.

Laboratory measurements. All clinical laboratory data for the type 2 DM (T2DM-1) and type 1 DM (T1DM) patients were obtained at the Central Clinical Laboratory of the University Hospital of Shiga University of Medical Science or the Central Clinical Laboratory of the University of Tokushima. Plasma glucose was measured by the glucose oxidase method. Insulin and urine C-peptide immunoreactivity (U-CPR) were estimated using ELISA methods, while the HbA1c level was measured by high performance liquid chromatography (HPLC). Total cholesterol, triglyceride, high-density lipoprotein (HDL)-cholesterol, free fatty acid, lactate and glycoalbumin levels were

determined using standard enzymatic methods.

Human IR-expressing transgenic mice. In this study, we used 2 strains of transgenic mice expressing human IR. (See details in Online Appendix)

Immunoblotting. Immunoblotting was carried out using specific antibodies as indicated in the legends and as previously described, except using Can-get-signal solution™ (Toyobo, Osaka, Japan) for primary antibody dilution(8).

Reagents. All other reagents were of analytical grade and obtained from Sigma or Nacalai Tesuque (Kyoto, Japan).

Ethical issues. All study protocols and designs were approved by the Ethics Committees of the University of Tokushima (approval#171) and/or the Shiga University of Medical Science (approval#16-36). We also obtained written consent from all participants who were given written information regarding the study. Animal experiments were also approved by the Animal Ethics Committees of the University of Tokushima (approval#16-57 & #05052) carried out in accordance with international care regulations.

RESULTS

Detection of soluble IR ectodomain in human plasma.

Based on the hypothesis that soluble IRα may exist in the human plasma, we attempted to detect IRα in human plasma. After partial purification by wheat-germ agglutinin (WGA)-conjugated affinity chromatography (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and immunoprecipitation with an anti-hIRα-specific antibody (5D9), IRα (135 kDa) was detected by immunoblotting with an IRα-specific antibody (N-20). A band corresponding to hIRα, but not the IR precursor, was observed and its peak signal intensity corresponded to the peak absorbance in hIRα ELISA analyses (A_{450}) (Fig. 1A). Furthermore, intact hIRβ was not detected by immunoblotting with an anti-IRβ antibody that recognized the C-terminal 19 amino acids of IRβ (data not shown), suggesting that the hIRα observed in human plasma was probably derived by the cleavage of the ectodomain (α-subunit and a part of β-subunit) of the receptor from cell surfaces, rather than by release from damaged tissues (cells) or microvesicles (see Online Appendix Fig. 3 and Table 1). Furthermore, we also compared the molecular weights of the soluble hIR ectodomain in human plasma with standard hIR-ectodomain protein (α-subunit and a part of β-subunit) (8). The standard protein was derived from CHO-IR-*SspI* cells(8) stably introduced the cDNA encoding whole human IR ectodomain except just 3 amino acids of peri-transmembrane region (amino acid 1-953) and its logical molecular weight was approximately 370 kDa (8). As shown in Fig. 1B, the retention time of immunoreactive hIR-ectodomain in human plasma was identical with that of standard hIR-ectodomain protein, suggesting that the molecular weight of both protein under non-reducing condition were almost same using Superdex-200 gel-filtration column. In addition, the apparent molecular weight was approximately 370 kDa. If the soluble hIR ectodomain existed as α-subunit homodimers, the molecular weight would be ~270 kDa. If the soluble hIRα exists with intact β-subunits as an intact hetero-tetramer, the molecular weight

would be ~460 kDa. Given all these data, the soluble hIRα appeared to exist with parts of the extra-cellular region of β-subunits, and proteolytic cleavage ("shedding") appeared to occur at a site in the extracellular peri-transmembrane region (Online Appendix Fig. 3).

Measurements of soluble IRα in human plasma from DM patients using the newly established hIRα-specific ELISA system.

Based on the above results, we established ELISAs for both hIRα and full-length hIR, that were highly accurate, specific and unaffected by either the presence of insulin or hemolysis (see details in the Method section). According to the receiver operated characteristic (ROC) analysis using the data of control-1 and T2DM-1 groups, the area under the curve was 0.794 ± 0.217 ($p<0.0001$, Online Appendix Fig. 1-G), suggesting that this hIRα ELISA system has significant specificity and sensitivity in separating these 2 groups. We also used a commercially available IRβ ELISA system, as well as a full-length IR ELISA, to confirm that the plasma hIRα was not derived from damaged cells or micro-vesicles. To investigate the clinical significance of the presence of IRα in human plasma, we measured the hIRα levels in samples from patients with DM. First, we examined the variations in the hIRα level in normoglycemic individuals throughout the day, and found that the level did not change in normoglycemic subjects, even in the postprandial state (Online Appendix Fig. 1F). Next, we measured plasma samples obtained from both type 1 DM (T1DM) and T2DM patients as well as from control subjects (Control-1, in Tokushima district) who had been confirmed to be normoglycemic by oral glucose tolerance tests according to the WHO criteria(10). As shown in Fig. 2A, the T2DM patients exhibited a significantly elevated plasma hIRα level compared with the control subjects [2.26 ± 0.80 (T2DM-1, n=474) vs. 1.59 ± 0.40 ng/ml (Control-1, n=123), $p<0.001$]. On the other hand, the levels of plasma intact IRβ and full-length IR were negligible in the T2DM plasma samples with a high hIRα level (Table 1), indicating that the plasma hIRα existed without intact IRβ and was derived from the cell surface by cleavage, rather than by release from damaged cells. These results are also supported by Fig. 1B. The plasma hIRα level was also increased in the T1DM patients (2.00 ± 0.60 ng/ml (T1DM, n=53), $p<0.001$ vs. Control-1; Fig. 2A) and the levels of plasma intact IRβ and full-length IR were also negligible (Table 1). More than 30% of the DM patients exhibited a higher hIRα level than the cut-off value (2.39 ng/ml; mean + 2 S.D. of Control-1). There were no differences in the hIRα levels among people from different districts. On a molar basis, the hIRα concentration in DM patients was about 20% of the fasting plasma insulin concentration. We also evaluated the percentages of insulin-bound hIRα in plasma from 11 cases of T2DM patients by measuring the changes in the immunoreactive insulin (IRI) level after depletion of hIRα by 5D9 (anti-hIRα)-affinity beads. Under conditions of almost complete hIRα-depletion, comparable amount (approximately 10-20%) of the IRI was depleted by the anti-hIRα-antibody beads. Therefore, at most 10-20% of the IRI binds to the plasma hIRα (Table 2).

Next, we analyzed the correlations of the hIRα level with the clinical parameters of the DM patients (Online Appendix Table 1). There were significant positive correlations between the hIRα level and the blood glucose level, including the fasting blood glucose ($p<0.001$) and HbA1c ($p<0.001$) levels (Online Appendix Table 1, Online Appendix Fig. 2A and 2B). On the other hand, the hIRα level was not correlated with markers of insulin secretion, including the fasting IRI level, 24-h urinary CPR excretion or homeostasis model for assessment of β-cell function (HOMA-β) value. The hIRα level also exhibited a very weak correlation with age ($p<0.001$) in the T2DM-1 patients, but not in the Control-1 and Control-2 subjects or T2DM-2 patients. As shown in Online Appendix Table 1, there were no correlations with other standard clinical parameters. Moreover, there were no significant differences in the hIRα levels among the types of treatments the patients were receiving. To further confirm the relationship between the hIRα level and the blood glucose level, we examined 18 patients whose HbA1c level had changed greatly (≥1.8%) within the last 3 years. As shown in Fig. 2B, there was a strong positive correlation between the changes in the plasma hIRα level and those in the HbA1c level ($N=18$, $p<0.001$, $R=0.84$). To examine whether hyperglycemia induced the increase in plasma hIRα, we followed the clinical courses of 8 new-onset T1DM patients (Fig. 2C), all of who were sent to the University Hospital of the University of Tokushima due to the onset of T1DM. At admission, the FPG, HbA1c and hIRα levels were 350 ± 82 mg/dl, 12.6 ± 2.1% and 5.5 ± 1.8 ng/ml, respectively. At discharge after 6-10 days of intensive insulin therapy, their blood glucose level had almost normalized (124 ± 31 mg/dl) and their hIRα level had decreased to 3.3 ± 1.0 ng/ml. After 1 month, the patients' glycemic control levels were maintained with insulin therapy and their HbA1c, glycoalbumin and hIRα levels were 9.2 ± 1.3%, 24.9 ± 2.4% and 2.3 ± 0.8 ng/ml, respectively. At that moment, only hIRα almost reached to plateau, but glycoalbumin and HbA1c levels were further decreased at 2 months. The results presented in Figs. 2B and 2C strongly suggest that the plasma hIRα level promptly parallels any changes in the blood glucose level and changed more rapidly than glycoalbumin or HbA1c. In addition, there was a significant positive correlation of the hIRα levels with the blood glucose levels including blood glucose, glycoalbumin and HbA1c levels (Online Appendix Table 1, Figs. 2D, Online Appendix 2A and Online Appendix 2B). The regression values were higher in T1DM (Fig. 2D) than in T2DM (Online Appendix Figs. 2A and 2B). Furthermore, during long-term follow up of a T1DM patient, the hIRα level completely paralleled with the glycoalbumin and HbA1c levels and it changed more rapidly and dynamically than the glycoalbumin and HbA1c levels (Fig. 2E).

Elevation of the plasma hIRα level in streptozotocin-induced diabetic mice transgenically expressing hIR.

To further confirm these clinical observations *in vivo*, we initially used TG mice that systemically express kinase-deficient human IR (hIR^{K1030M}TG) (11; 12). Although these TG mice express a kinase-deficient mutant human IR (K1030M; Lys→Met at residue 1030 in the kinase domain of the β-subunit), they do not show diabetic phenotypes (11; 12).

Furthermore, the mutation did not appear to affect the receptor release based on analyses of cultured cells (data not shown). In addition, we recently generated and analyzed WT-hIR-expressing TG mice that systemically expressed at least 4-fold higher amount of hIR than hIR^{K1030M}TG mice (data not shown).

DM was induced in either TG or control mice (non-transgenic littermates) (NTG) by intraperitoneal streptozotocin (STZ) injection. After 4-7 days, diabetic hIR-TG mice showed a markedly higher human IRα level, and this was strongly correlated with their blood glucose level ($N=14$, $p<0.001$, $R=0.80$; Fig. 3C). On the other hand, NTG diabetic mice or non-diabetic human IR-TG mice showed negligible hIRα levels, similar to NTG non-diabetic mice. In the NTG diabetic mice, the endogenous mouse IRα level was expected to increase, but was not detected with the human IRα-specific ELISA systems. The level of full-length IR was also confirmed to be negligible using these ELISA systems, indicating that the plasma hIRα was not derived from damaged cells (Fig. 3B). Furthermore, to investigate whether the IRα responses were attributed to the changes of glucose level, we treated the STZ-induced diabetic mice with insulin (twice a day subcutaneous injection of neutral protamine Hagedorn (NPH) human insulin (Novolin-N, NovoNordisk, Bagsværd, Denmark)). As shown in Fig. 3D, the IRα levels were promptly decreased by insulin therapy paralleled with blood glucose level. Moreover, after the transient pause (3days) of insulin treatments, both blood glucose and IRα levels were re-elevated, and then promptly re-declined by the resume of insulin therapy. Notably, some mice failed to get diabetes even with same dose of STZ-injection, and did not exhibit the elevation IRα levels. We also treated non-diabetic mice with sustained release insulin implants (Linshin Canada In., Toronto, Ontario, Canada), and these mice showed normoglycemia and marked weight gain, but the IRα levels were changed little (data not shown), indicating that neither STZ nor insulin alone did change the plasma IRα levels. We further estimated the half-life of the IRα in blood using the WT-hIR-TG mice. After the diabetic induction by STZ, insulin therapy was initiated and plasma IRα levels were followed. As shown in Fig. 3E, the circulating plasma IRα levels were decreased to $49.3 \pm 13.8\%$ ($N=9$) at 6 h after the treatment, $28.7 \pm 17.5\%$ ($N=19$) at 24 h, and $19.0 \pm 12.3\%$ ($N=19$) at 48h.

DISCUSSION

In this study, we have provided evidence that soluble hIR ectodomain (α-subunit and a part of β-subunit), but not intact hIRβ or whole hIR, exists in human plasma. Furthermore, patients with T2DM, as well as those with T1DM, showed a significantly elevated plasma hIRα level compared to control subjects, as measured using newly established human IR-specific ELISA systems (i.e., hIRα-specific and whole hIR-detectable systems). The plasma hIRα level was positively correlated with the blood glucose, glycoalbumin and HbA1c levels. In addition, comparable amount of plasma insulin appeared to bind to hIRα. Moreover, hyperglycemia was confirmed to induce human IRα release in streptozotocin-induced diabetic mice transgenically expressing human IR.

The ectodomains of receptors for several cytokines and growth factors have

been found to circulate in the plasma (1-3). The existence of soluble IR in human plasma has previously been suspected, since several studies have reported shedding of IR from cultured cells (e.g., IM-9 human lymphoblasts, MCF-7 human breast cancer cells, HepG2 human hepatoma cells and human lymphocytes, as well as 3T3-L1 mouse fibroblasts transfected with human IR) (4; 6; 7). We have also observed hIRα release from CHO-hIR, HepG2 cells, hIR-expressing L6 myocytes and hIR-expressing 3T3-L1 adipocytes (Obata et al. unpublished data).

Although Pezzino *et al.* reported the detection of both IRβ and IRα in healthy human plasma that presented insulin-stimulated autophosphorylation activity without tyrosine kinase activity against exogenous substrates (5), we did not observe a protein corresponding to the β-subunit, at least in its intact form, in either of the ELISA (full-length and β-subunit ELISA systems) or the immunoblotting (data not shown). We showed that IRα is released concomitantly with part of the extracellular domain of IRβ (Online Appendix Fig. 3) into the plasma of DM patients using Superdex gel-filtration column (Fig. 1B). In fact, many membrane proteins (e.g., TNF receptors, EGF receptor and IL-6 receptor) have soluble ectodomains that are usually cleaved at a site in the stalk region between the transmembrane segment and the globular extracellular domain. The distances of the cleavage sites from the plasma membrane are approximately 1-43 amino acids (13). In accordance with these report, as shown in Fig. 1B, the retention times of immunoreactive soluble hIR in human plasma and standard hIR ectodomain protein derived from CHO-IR-*SspI* cells were similar, suggesting that the molecular weight of both protein under the non-reducing condition were almost same. Moreover, the apparent molecular weight was approximately 370 kDa. Considering all these information, the soluble hIRα in human plasma appeared to exist with parts of the extra-cellular region of β-subunits, and the shedding appeared to occur at a site in stalk region in extracellular peri-transmembrane region, like the cases of other soluble receptors(13). (Online Appendix. Fig. 3). Since the actual cleavage site is unclear at this time, further experiments are necessary. Recently, the crystal structure of IR-ectodomain has been solved (14), so these informations must be helpful.

In the present study, the clinical results indicated that the release of hIRα into the plasma may be augmented by hyperglycemia, and the subsequent *in vivo* study using hIR-transgenic mice supported these findings. On the other hand, the plasma hIRα level was not correlated with markers of insulin secretion, suggesting that the release of hIRα into the plasma may be regulated by the blood glucose level, rather than by the secreted insulin. The concentration of hIRα in patients with DM was, on average, approximately 20% (20.3 $\pm$ 51.2%, N=88) of the fasting IRI levels on a molar basis. Comparable amount (approximately 10-20%) of insulin was immunodepleted by anti-IRα-antibody (Table II), suggesting that appreciable amount of the plasma insulin appeared to bind to plasma soluble hIRα. In turn, the absolute amount of plasma insulin sequestered by hIRα in DM patients seemed to be much larger than that in normoglycemic subjects. In case of autoantibody to insulin, the antibody first sequesters insulin, and then releases insulin

after a time(15). So the apparent insulin-bound hIRα fraction may be underestimated. In addition, although we expected that the released soluble IR possibly participates in insulin-resistance as one of the factors that contribute to glucose toxicity by sequestering plasma insulin, we found no correlations of the hIRα level with other parameters reflecting insulin-resistance (i.e., HOMA-IR) as well as fasting IRI level in the present study.

According to clinical data (Fig. 2C and 2E), the hIRα level changed more rapidly and dynamically than HbA1c or glycoalbumin levels. So, we estimated the half-life ($T^{1/2}$) of hIRα using STZ-induced diabetic TG mice (Fig. 3E). The $T^{1/2}$ was estimated to ~6h and it was much shorter than that of HbA1c ($T^{1/2}$: 30 days) or glycoalbumin ($T^{1/2}$: ~17 days), suggesting that IRα could be a more rapid glycemic marker. We examined the daily profile of hIRα levels and observed little change throughout the day (Online Appendix Fig. 1F) in normoglycemic subjects. However, considering such a short half-life, daily change may be able to be observed in patients with diabetes, especially with brittle diabetes. Furthermore, the shedding of IR appeared to need biological response to hyperglycemia, while HbA1c or glycoalbumin are elevated in response to hyperglycemia simply by non-enzymatic mechanisms. Thus, the plasma hIRα levels may reflect biological response to hyperglycemia.

Many membrane proteins have soluble ectodomains that are subject to proteolytic release, i.e., the process known as shedding(16). In most cases, shedding is caused via proteolytic cleavage by members of the ADAM family of membrane-tethered zinc metalloproteinases (MMPs) (16). We have also observed suppressive effects of a general MMP inhibitor (i.e., GM-6001) on IRα shedding (Obata et al. manuscript in preparation), suggesting the involvement of MMPs in this process. Most MMPs have C-terminal cytoplasmic tails that have been shown to be phosphorylated by various protein kinases including protein kinase C (PKC) (17; 18). This phosphorylation subsequently activates the protease activity(17; 18). Under high glucose conditions, the activation of PKC by *de novo* synthesis of diacylglycerol is well known(19; 20), including in endothelial cells(21) that also express IR(22). All these data suggest a vicious cycle for the glucose toxicity; from high glucose stimulating PKC activation, which in turn activates MMPs that cause the release of soluble IR into the plasma, which sequesters insulin, thereby further raising glucose levels. The latter hypothesis is supported by our prior studies showing that the injection of purified hIRα into mice elevated blood glucose levels *in vivo*(8). At this time, the source organ of soluble IR is unclear. Noteworthy, although we detected plasma soluble IR in hIR-Tg mice, the expression level of IR in the liver was extremely lower than that in other organs.

Regarding the shedding of other membrane proteins in the diabetic state, Lim et al. reported that soluble CD40 ligand and soluble P-selectin levels were increased in plasma from patients with DM, and they proposed that the increments promoted atherothrombotic complications in cardiovascular disease (23). In the diabetic condition, other receptor proteins as well as IR are possibly shed from the cell membrane. However, the shedding of these membrane proteins except IR was not associated with glycemic control. In our hand, we measured plasma soluble

TNFα-R1 and soluble IL-6R levels, and compared them with plasma hIRα level (Online Appendix Fig. 4A-4B). The plasma soluble TNFα-R1, but not soluble IL-6R, levels significantly correlated with the hIRα levels ($P<0.001$), suggesting that there may exist both common and distinct mechanisms between the shedding of IR and these receptors. Thus, the possible existence of various mechanisms for shedding needs to be considered.

In general, the physiological roles of ectodomain shedding of membrane receptors were diverse and complex. In the case of growth hormone receptor (GHR), the shedding of GHR generate GH binding protein that interact with GH with high affinity and therefore down-regulates the availability of the ligand (24). In this way, the majority of soluble receptor ectodomain by shedding appeared to inhibit the actions of their ligands (25), but soluble IL-6 receptor seems to act agonistically upon IL-6 binding (26). In contrast, in the case of TrkA (the receptor for nerve growth factor), after the ectodomain shedding, residual membrane-associated fragments were found to be phosphorylated, associated with intracellular signaling molecules and potentiated its action (27). In this case, the process appeared to play a compensative positive role. In fact, expression of ectodomain-truncated IR has also been shown to exhibit ligand-independent activation (28). At present, the physiological role of the process of IR ectodomain shedding remains unclear. Even in normoglycemic subjects, some basal hIRα level was detected. To clarify the issue, further study must be necessary.

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Abbreviations footnote: IR: insulin receptor, hIRα: human insulin receptor α-subunit, DM: diabetes mellitus, T1DM: type 1 diabetes mellitus, T2DM: type 2 diabetes mellitus, WGA: wheat-germ agglutinin, IRI: immunoreactive insulin, HOMA-IR: homeostatic model assessment scores for insulin-resistance, HOMA-β: homeostasis model for assessment of β-cell function, MMP: membrane-tethered zinc metalloproteinase, GH: growth hormone, FPG: fasting plasma glucose, CPR: C-peptide immunoreactivity, HPLC: high performance liquid chromatography, HDL: high-density lipoprotein, STZ: streptozotocin, TMB: tetramethylbenzidine, CMV: cytomegarovirus
CI: coefficient interval

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Table 1

Comparisons of the plasma hIRα, intact IRβ and full-length hIR levels in patients with diabetes mellitus (DM).

The plasma hIRα, intact IRβ and full-length hIR levels in patients with type 2 DM (T2DM) or type 1 DM (T1DM) whose hIRα levels were relatively higher than that of the control subjects are shown. All the ELISAs were normalized by a common full-length IR standard protein, and the titer of each sample is indicated as the hIRα content (ng/ml).

	hIRα (ng/ml)	Intact IRβ (ng/ml)	Full-length hIR (ng/ml)
T2DM (high hIRα)	4.37	0.00	0.34
	4.47	0.00	0.25
	4.49	0.00	0.15
	4.75	0.00	0.10
	4.79	0.00	0.22
	4.95	0.00	0.00
	5.04	0.00	0.06
	5.31	0.12	0.00
	6.52	0.00	0.00
	8.07	0.34	0.00
T1DM (high hIRα)	8.80	0.44	0.02
	7.49	0.00	0.22
	9.15	0.26	0.05
	6.39	0.16	0.04
	5.52	0.26	0.01
	6.08	0.48	0.05

Table 2

Insulin binding by hIRα in human plasma from patients with T2DM.
Aliquots (50 μl) of human plasma samples from 11 T2DM patients were diluted with 100 μl of insulin-binding buffer (50 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 1% BSA, 0.1% Tween 20) and immuno-depleted with 5D9 (an anti-hIR-specific monoclonal antibody)-protein A-Sepharose beads for 16 h at 4°C with gentle agitation. The depleted aliquots and residual non-depleted samples were analyzed using both insulin (Mesacup Insulin ELISA kit; MBL, Nagoya, Japan) and hIRα ELISA systems. Binding of hIRα to insulin did not affect the insulin ELISA results (data not shown). Non-specific binding of insulin to 5D9-protein A-Sepharose beads is confirmed to be negligible (data not shown). The percentages of residual insulin and hIRαwere calculated, and immuno-depleted IRI was considered as apparent hIRα-bound IRI. IRI: immunoreactive insulin.

	Pre-5D9 depletion		Post-5D9 depletion		% residue		Apparent hIRα-bound IRI
	IRα (ng/ml)	IRI (μU/ml)	IRα (ng/ml)	IRI (μU/ml)	IRα (%)	IRI (%)	IRI (%)
T2DM#1	1.92	59.3	0	53.4	0	90.1	9.9
T2DM#2	2.03	49.9	0.04	43.8	2.0	87.8	12.2
T2DM#3	1.27	14.3	0.02	12.1	0.9	84.6	15.4
T2DM#4	1.90	12.3	0	9.9	0	80.5	19.5
T2DM#5	1.29	9.8	0	8.6	0	87.8	12.2
T2DM#6	0.81	185.0	0	160.0	0	86.6	13.4
T2DM#7	2.78	25.8	0	22.3	0	86.4	13.6
T2DM#8	2.46	16.5	0	13.3	0	80.6	19.4
T2DM#9	2.68	17.5	0	15.5	0	88.6	11.4
T2DM#10	2.02	53.3	0	46.6	0	87.6	12.4
T2DM#11	2.12	42.7	0	36.2	0	84.8	15.2

Figure Legends

Figure 1. (A) Detection of soluble IRα in human plasma. A 10 ml sample of human plasma (hIRα: ~5 ng/ml) obtained from a patient was diluted to 50 ml with PS buffer (20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl) and applied to a WGA-column (2 ml bed volume). The column was washed with PS buffer, and eluted with the same buffer supplemented with 0.3 M N-acetyl-glucosamine. Aliquots (300 μl) of the 1 ml fractions obtained were subjected to immunoprecipitation (IP) with the 5D9 antibody, and analyzed by immunoblotting (IB, reducing condition) with an anti-IRα antibody (N-20). The titer of hIRα in each fraction obtained from the WGA-column chromatography was assayed by the hIRα ELISA and is shown as the absorbance at 450 nm (A_{450}). **(B) Comparison of the native molecular weights of human plasma soluble IR and standard IR-ectodomain protein using Superdex 200 column.**
A 0.5 ml sample of human plasma obtained from a normal subject and standard hIR ectodomain protein for the ELISA system were diluted to 0.5 ml with column buffer (10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% BSA and 0.1% NaN_3) and applied to a Superdex 200 gel-filtration column (1.5 x 60 cm, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The column was eluted with same column buffer. Aliquots (100 μl) of the 1.0 ml fractions obtained were subjected to human IRα-specific Sandwich ELISA system. The titer of hIRα (ng/ml) and the absorbance at 280 nm (A_{280}) in each fraction obtained from either human plasma (closed circle) or standard hIR-ectodomain protein (closed triangle) are shown. The approximate molecular weight of IgG (150 kDa) and albumin (67 kDa) in human plasma sample were also shown. The eluted fractions showed the analytical recovery of immunoreactive plasma soluble hIR and standard hIR ectodomain to be 87% and 97%, respectively.

Figure 2. Plasma hIRα levels in patients with type 1 and type 2 diabetes mellitus. (A) Comparisons of the plasma IRα levels among patients with diabetes mellitus (DM) and normoglycemic control subjects. T2DM-1: outpatients with T2DM seen at the University Hospital of Shiga University of Medical Science (N=474); T2DM-2: outpatients with T2DM seen at the University of Tokushima Affiliated Hospital (N=162); T1DM: outpatients with T1DM seen at the University of Tokushima Hospital (N=53); Control-1: healthy non-diabetic volunteers from the University of Tokushima (N=123) who were confirmed to be normoglycemic by oral glucose tolerance tests; Control-2: healthy non-diabetic volunteers from Medical & Biological Laboratories Co. Ltd (N=120) who were confirmed to have normal fasting plasma glucose (FPG) and HbA1c levels. Data are expressed as the mean ± SD. The cut-off value (2.39 ng/ml) was determined by the mean + 2 S.D. of the Control-1 value. ***: $p<0.001$ by Student's *t*-test. (B) Scatter plot showing the correlation between changes in the plasma hIRα level and changes in the HbA1c level over 36 months (March 2000-March 2003) in selected (ΔHbA1c≥1.8) T2DM-1 patients (N=18). The correlation coefficient was determined by Pearson's correlation coefficient test. (C) Clinical courses of 8 new-onset T1DM patients. The hIRα, FPG GA

(glycoalbumin) and HbA1c levels (mean ± S.D.) at admission, discharge (6-10 days after admission), 1-month and 2-months follow-up are shown. (D) The scatter plot showing correlation of plasma hIRα level with HbA1c, glycoalbumin and the plasma glucose in 118 samples from 64 type 1 DM patients. The correlation coefficients were determined by Peason's correlation coefficient test for glycoalbumin, plasma glucose and HbA1c. (E) The serial changes of glycemic control markers in a T1DM patient. The glycemic markers (HbA1c; closed square, glycoalbumin; open square, hIRα; open triangle) of an outpatient of T1DM seen at the University of Tokushima Hospital were followed. GA; glycoalbumin.

Figure 3. Detection of hIRα in streptozotocin-induced diabetic mice expressing human insulin receptor. (A, B) Streptozotocin (STZ; 4 mg/20 g body weight) was injected intraperitoneally into 6-week-old fasted TG mice systemically expressing kinase-deficient human insulin receptor ($hIR^{K1030M}TG$) (29) or control non-transgenic littermates (NTG, C57BL/6 strain). After 4 - 7 days, blood samples were obtained and analyzed by the hIRα (A) and full-length IR (B) ELISA systems. Closed circles: STZ-injected mice; open circles: vehicle (50 mM citrate)-injected mice. Mice with a glucose level of ≥300 mg/dl were assigned to a diabetic group. ***: $p<0.001$ vs. the other groups by Bonferroni-Duncan's multiple comparison test. (C) Scatter plot showing the correlation between the plasma hIRα level and the blood glucose level in STZ-induced hIR-TG mice ($hIR^{K1030M}TG$). Closed circles: STZ-injected mice; open circles: vehicle-injected mice. The correlation coefficients were determined by Pearson's correlation coefficient test. **(D)** The serial changes of STZ-induced hIR-WT-TG mice with insulin treatment. Three days after the induction of DM with STZ, the mice were treated with NPH insulin (6-14 units/day, twice a day subcutaneous injection), then with 3 days transient pause of insulin treatments, followed by the resume of insulin therapy. Blood glucose levels were monitored, twice a day and the insulin doses were determined by glucose levels. (E) The estimation of half-life of plasma hIRα;β in plasma. Three days after the induction of DM with STZ, the diabetic hIR-WT-TG mice were treated with NPH insulin as above (6-14 units/day, twice a day subcutaneous injection). Circulating plasma hIRα;β levels were monitored at 0, 6, 24 and 48 h after initiation of insulin therapy.

Figure 1A

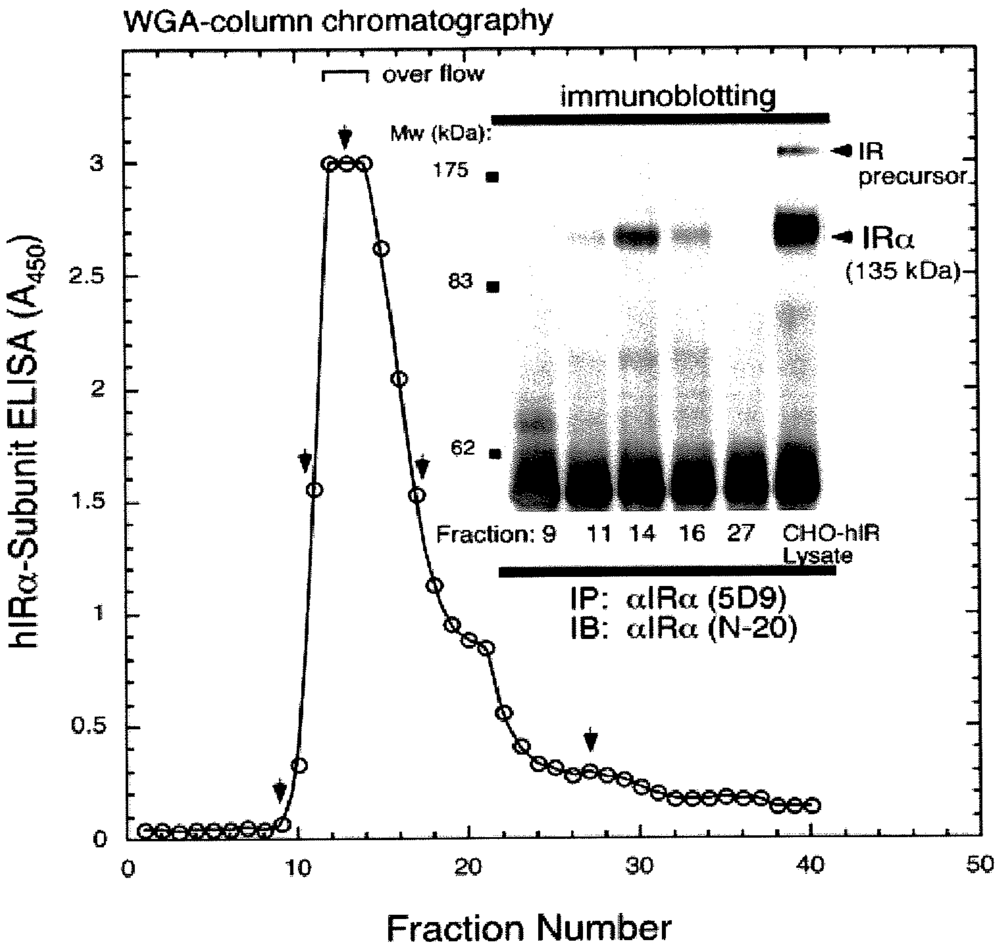

WGA-column chromatography
over flow
immunoblotting
Mw (kDa):
175
83
62
IR precursor
IRα (135 kDa)
Fraction: 9 11 14 16 27 CHO-hIR Lysate
IP: αIRα (5D9)
IB: αIRα (N-20)
hIRα-Subunit ELISA (A_{450})
0
0.5
1
1.5
2
2.5
3
0 10 20 30 40 50
Fraction Number

Figure 1B

Fig. 1B, Obata et al.

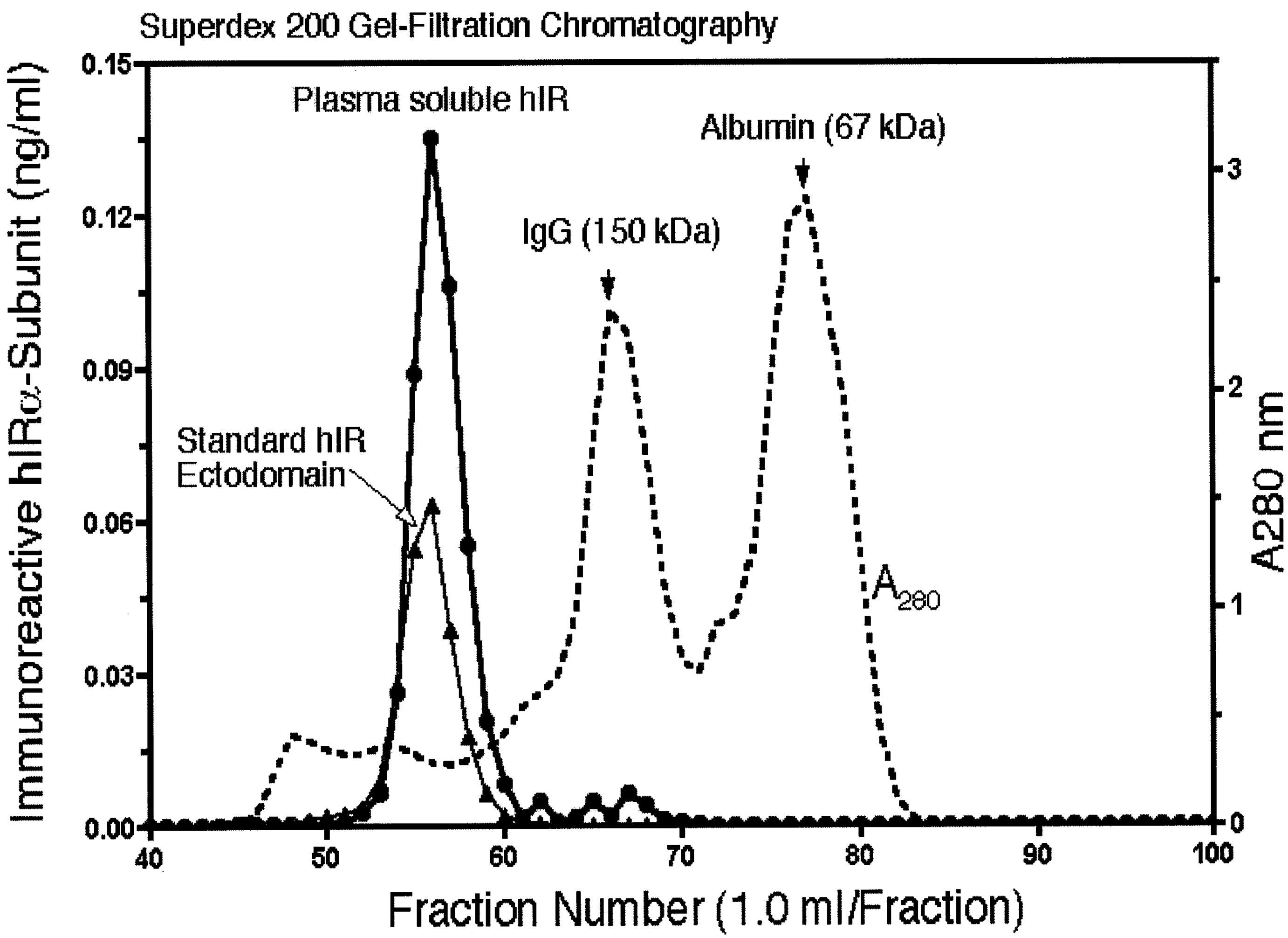


Figure 2A

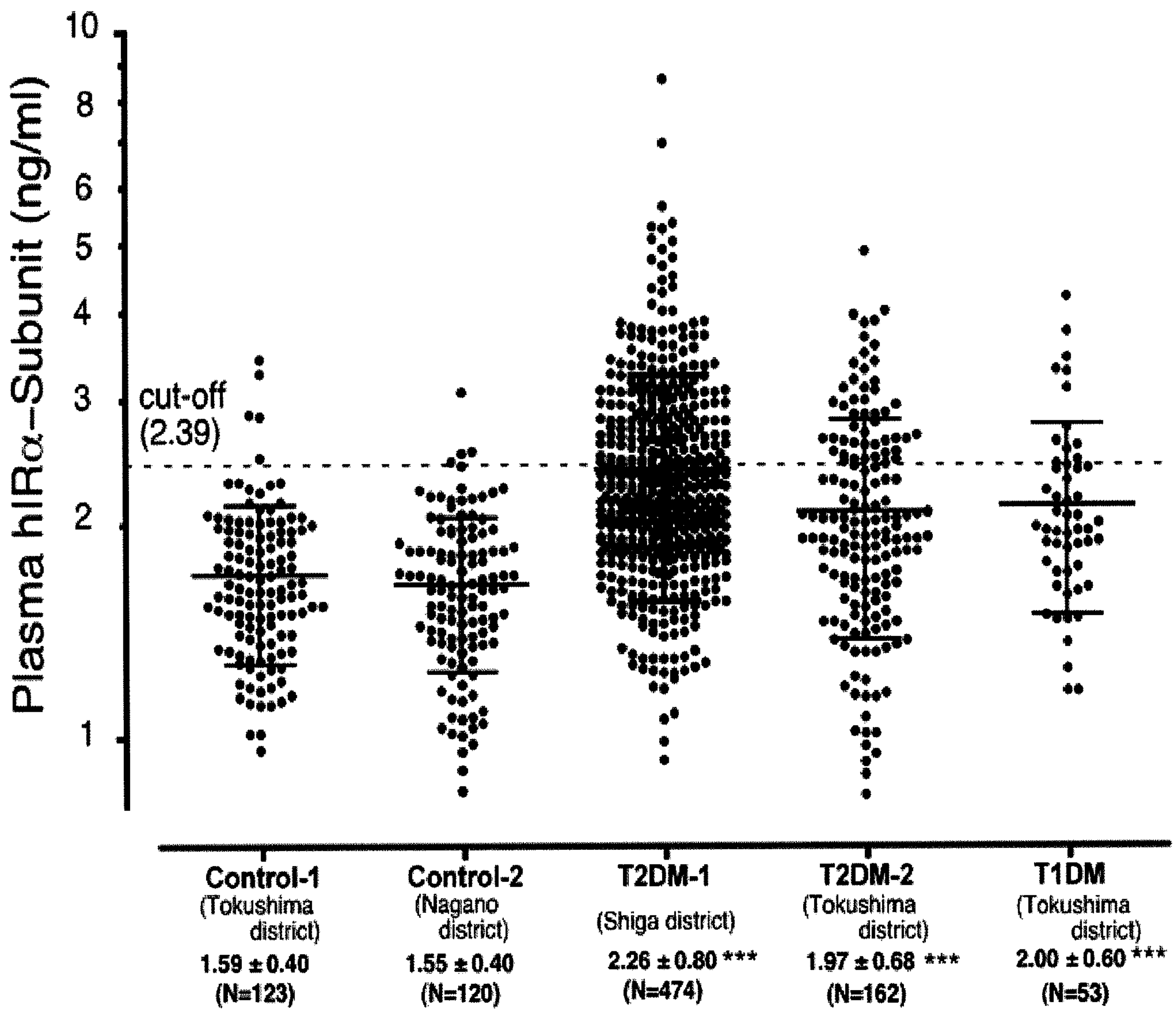
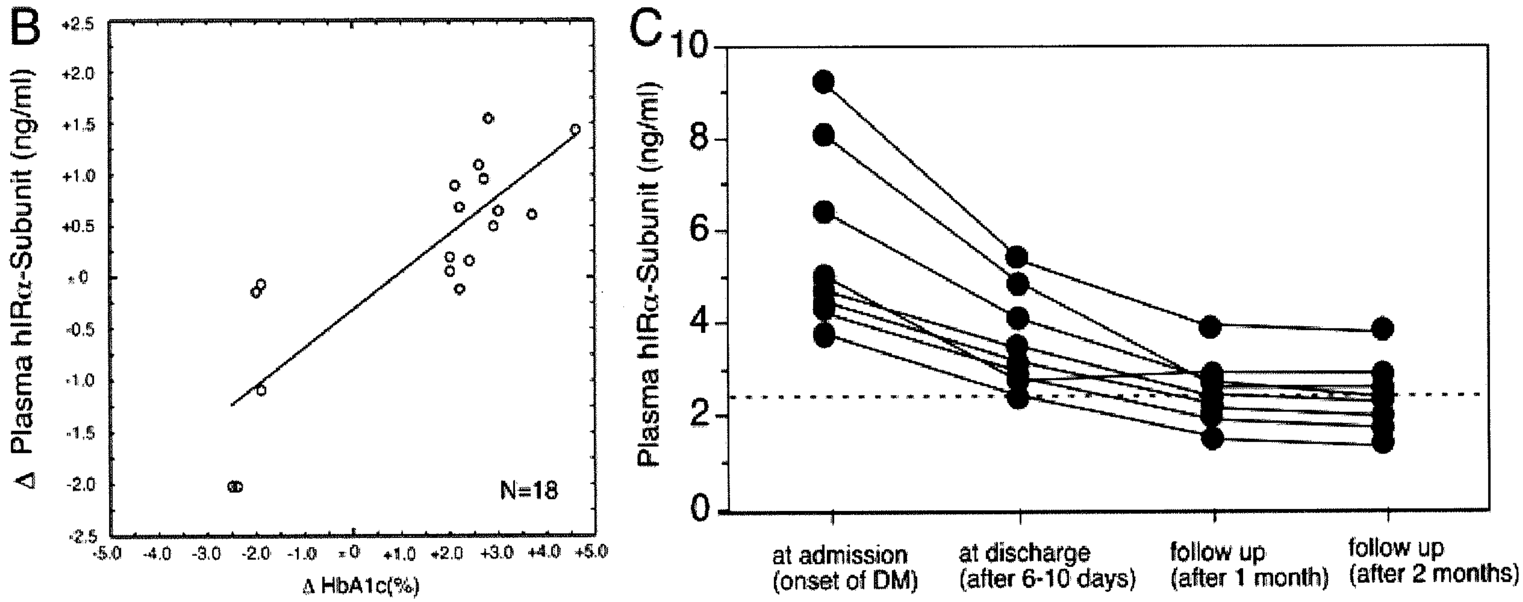

Plasma hIRα–Subunit (ng/ml)
10
8
6
5
4
3
2
1
cut-off
(2.39)
Control-1
(Tokushima district)
1.59 ± 0.40
(N=123)
Control-2
(Nagano district)
1.55 ± 0.40
(N=120)
T2DM-1
(Shiga district)
2.26 ± 0.80 ***
(N=474)
T2DM-2
(Tokushima district)
1.97 ± 0.68 ***
(N=162)
T1DM
(Tokushima district)
2.00 ± 0.60 ***
(N=53)

Figure 2B&C



	at admission (onset of DM)	at discharge (after 6-10 days)	follow up (after 1 month)	follow up (after 2 months)
FPG (mg/dl)	350 ± 115	124 ± 31		
GA (%)	44.8 ± 6.4		24.9 ± 2.4	18.9 ± 3.4
HbA1c (%)	12.6 ± 2.1		9.2 ± 1.3	6.9 ± 1.0
hIRα (ng/ml)	5.5 ± 1.8	3.3 ± 1.0	2.3 ± 0.8	2.2 ± 0.8

Figure 2D

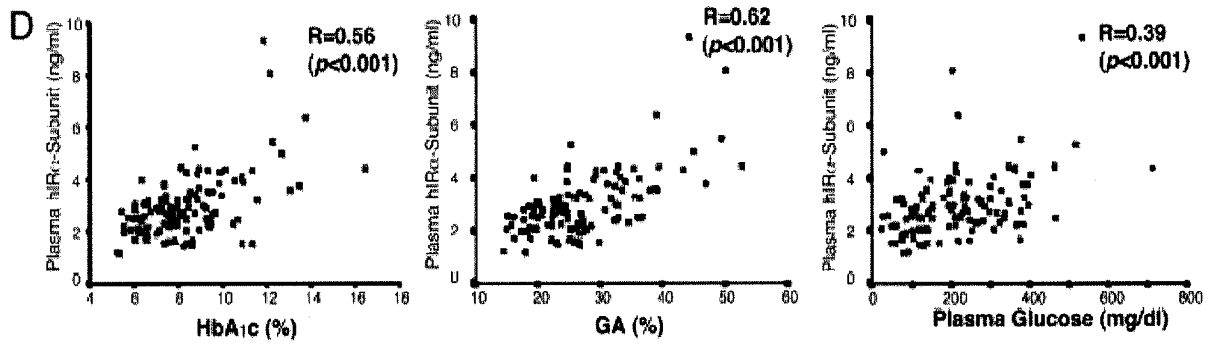

D
R=0.56
(p<0.001)
Plasma hIRα-Subunit (ng/ml)
HbA1c (%)
R=0.62
(p<0.001)
GA (%)
R=0.39
(p<0.001)
Plasma Glucose (mg/dl)

Figure 2E

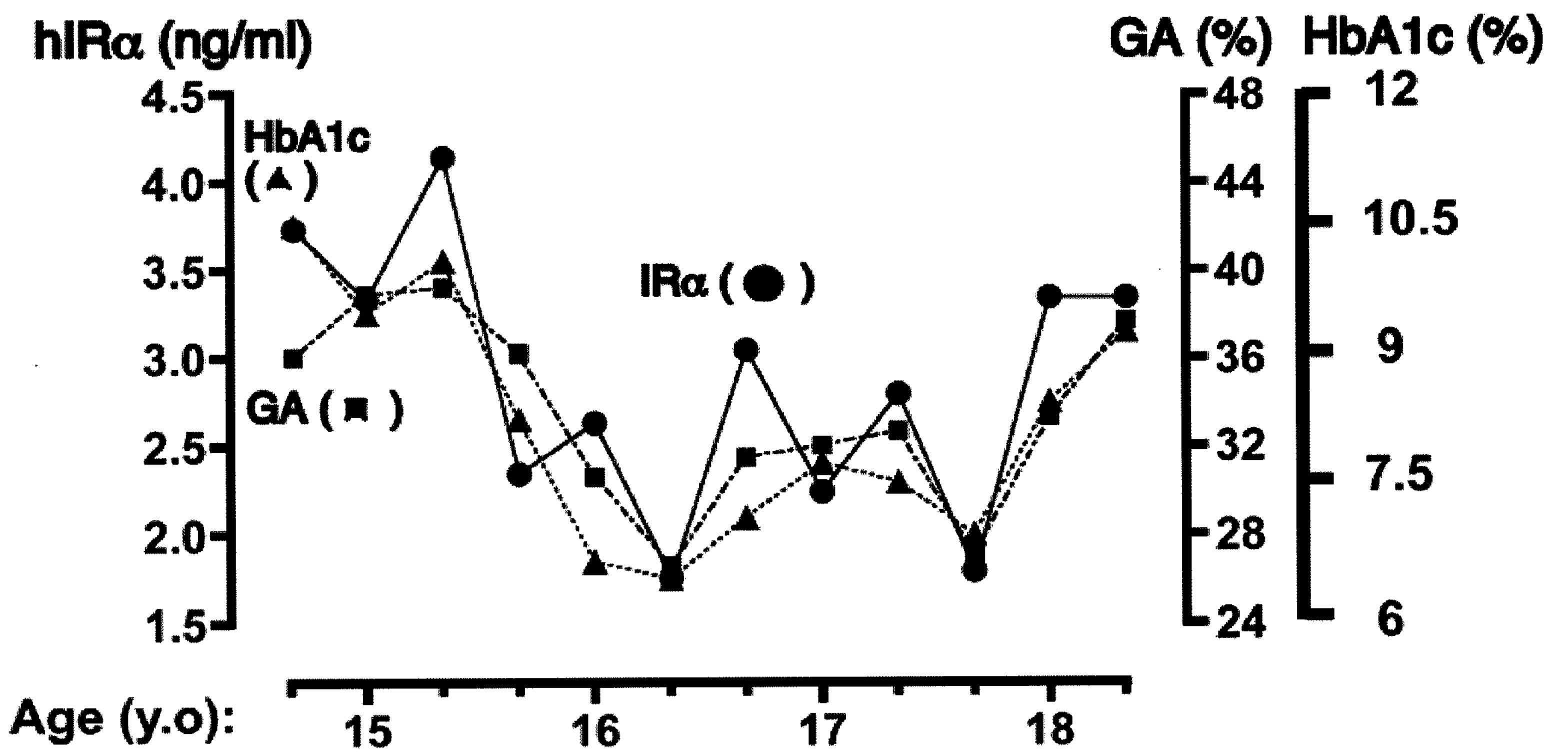

hIRα (ng/ml)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
HbA1c (▲)
GA (■)
IRα (●)
GA (%)
48
44
40
36
32
28
24
HbA1c (%)
12
10.5
9
7.5
6
Age (y.o):
15
16
17
18

Figure 3A, B&C

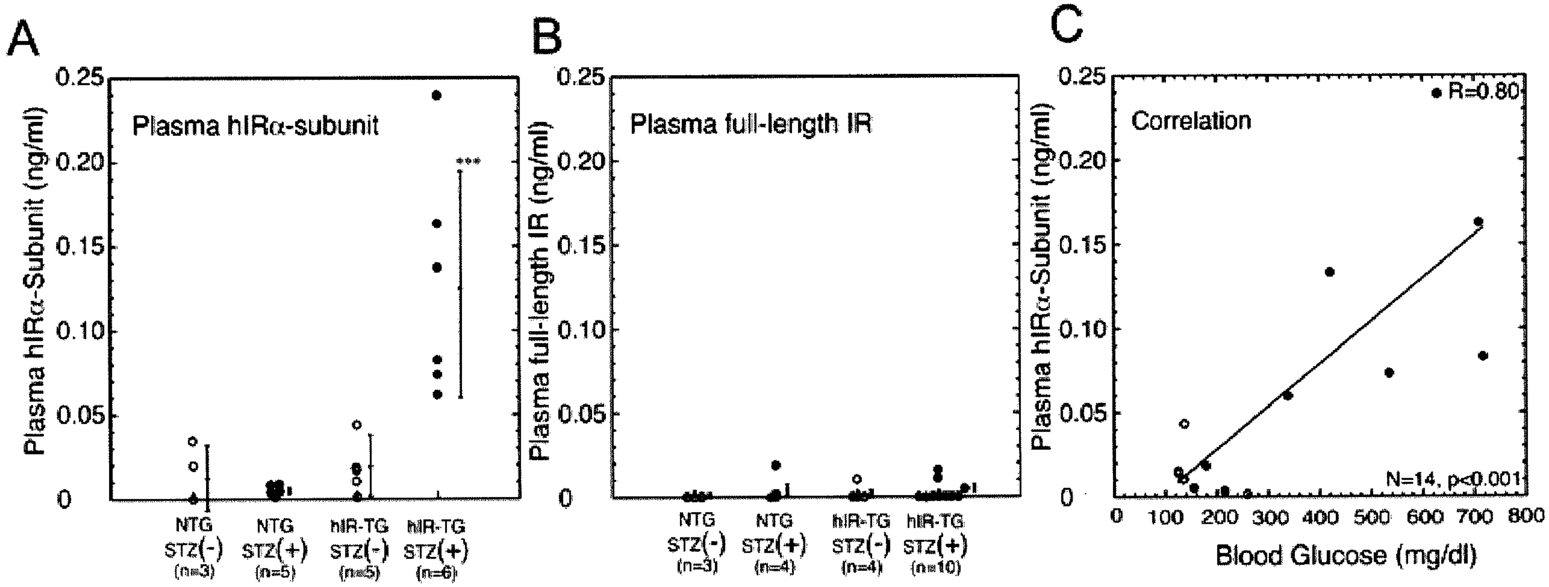

A
Plasma hIRα-subunit
Plasma hIRα-Subunit (ng/ml)
0.25
0.20
0.15
0.10
0.05
0
NTG STZ(-) (n=3)
NTG STZ(+) (n=5)
hIR-TG STZ(-) (n=5)
hIR-TG STZ(+) (n=6)
***
B
Plasma full-length IR
Plasma full-length IR (ng/ml)
0.25
0.20
0.15
0.10
0.05
0
NTG STZ(-) (n=3)
NTG STZ(+) (n=4)
hIR-TG STZ(-) (n=4)
hIR-TG STZ(+) (n=10)
C
Correlation
R=0.80
N=14, p<0.001
Plasma hIRα-Subunit (ng/ml)
0.25
0.20
0.15
0.10
0.05
0
0 100 200 300 400 500 600 700 800
Blood Glucose (mg/dl)

Figure 3D

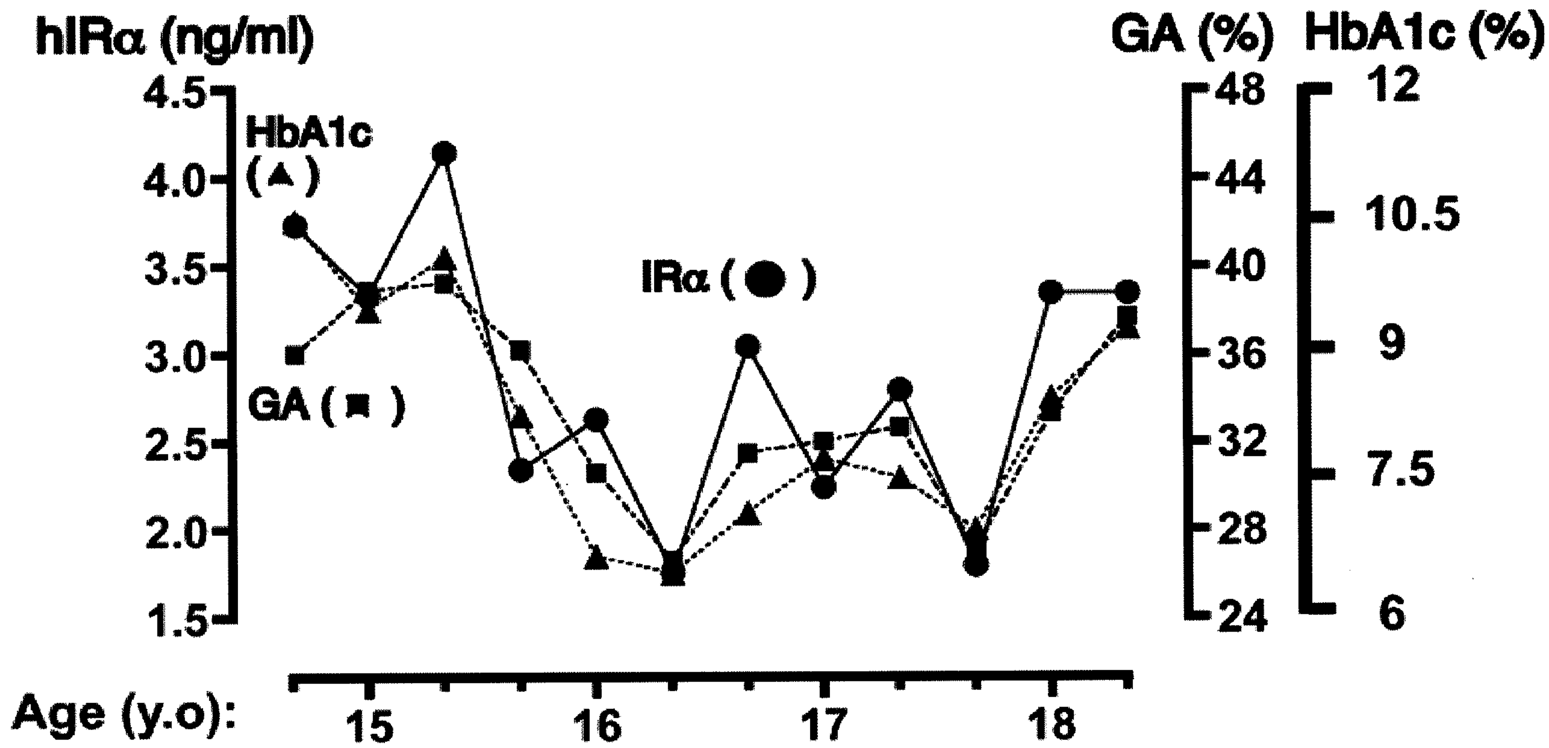
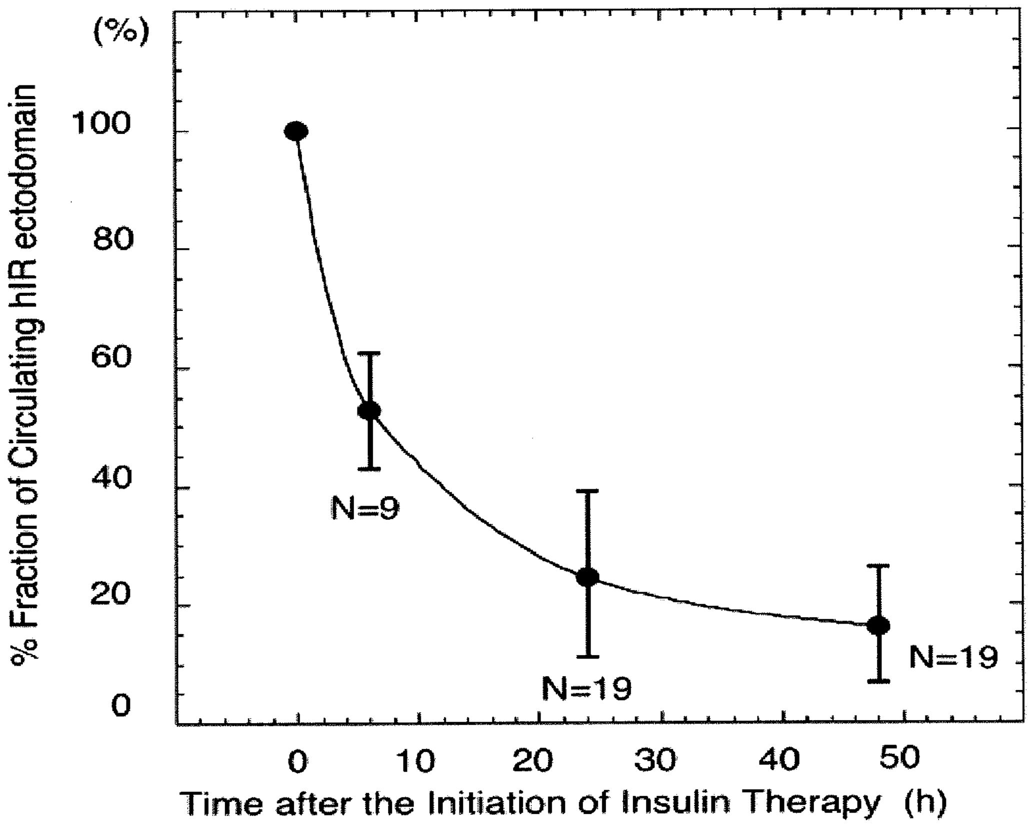

hIRα (ng/ml)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
HbA1c (▲)
GA (■)
IRα (●)
GA (%)
48
44
40
36
32
28
24
HbA1c (%)
12
10.5
9
7.5
6
Age (y.o):
15
16
17
18

Figure 3E


(%)
% Fraction of Circulating hIR ectodomain
100
80
60
40
20
0
N=9
N=19
N=19
0
10
20
30
40
50
Time after the Initiation of Insulin Therapy (h)